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13. ABSTRACT *(Maximum 200*

This proposal investigated the influence of diet on CCAAT/Enhancer binding proteins (C/EBPs) in mammary tumors and normal mouse tissue. Body weights of mice fed calorically restricted diets were lower than ad libitum fed mice. Body weights of mice fed high fat diets were greater than mice fed equicaloric low fat diets, suggesting that fat may be a more efficient source of dietary energy than carbohydrate. Diets did not alter mammary tumor incidence.

C/EBPs (-beta and -delta) are predominately localized to the nucleus in mammary tumors. Mammary tumors express abnormally high levels of a truncated C/EBP-beta translation product which may function in mammary tumor development. Diet did not affect C/EBP-beta translation products in mammary tumors, however, diet did affect C/EBP-alpha translation products in the liver. Restricted fed mice expressed both the full length (43kd) and the truncated (30kd) form of C/EBP-alpha. In contrast, ad libitum fed mice expressed high levels of the truncated C/EBP-alpha translation product. This finding may have important implications in the differing roles of C/EBP-alpha in growth control in liver. Nuclear extracts from mammary tumors bind to C/EBP consensus sites. The complex binding to the C/EBP consensus site appears to include C/EBP-beta and C/EBP-delta, but not C/EBP-alpha.

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PI - Signature Date

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5. INTRODUCTION

a. Summary of work: The work carried out in this proposal investigated the influence of diet on CCAAT/Enhancer binding proteins (C/EBPs) in mammary tumors in MMTV/c-neu transgenic mice and normal mouse tissue. C/EBPs are a family of DNA binding proteins implicated in the control of energy metabolism, growth and differentiation (1-10). Earlier studies in our laboratory had established that mammary tissue expresses a unique pattern of C/EBP isoforms (11). The MMTV/c-neu transgenic mouse was chosen as a model because female MMTV/c-neu mice develop invasive mammary intraductal adenocarcinomas (IDA) that undergo metastasis (12). These unique characteristics make the results from experiments with MMTV/c-neu mice highly relevant to human breast cancer as c-neu over expression, development of IDAs, and metastasis are common in human breast cancer (12-14).

b. General Introduction: Breast cancer is the number one form of cancer and the second leading cause of cancer deaths among US women (15). Of the known breast cancer risk factors (age, sex, diet, family and reproductive history) only diet could be realistically modified on a population-wide basis to reduce breast cancer incidence (16). National health agencies recommend a general reduction in fat intake to reduce breast cancer risk (16,17,19). Clinical trials with very low fat diets are in progress with high risk women and breast cancer patients (18,19). In addition, the historic Women's Health Initiative will test low vs high fat diets in a 10 year study enrolling 70,000 women (17-19).

Despite these efforts the "diet/breast cancer" hypothesis remains controversial (17). What is lacking is a precise definition of the link between diet and breast cancer. Without a biochemical mechanism, dietary recommendations will continue to be relatively nonspecific (ie, decrease fat intake). In addition, our limited understanding prevents any determination regarding which women would benefit most from strict adherence to dietary modification, and when these modification should be implemented to maximize their preventative effects. Our hypothesis is that diet acts as a mammary tumor promoter by inducing alterations in CCAAT/Enhancer binding proteins (C/EBPs). C/EBPs are a unique family of transcription factors implicated in the control of genes involved in energy metabolism, cell growth and differentiation (1-10). Aberrant expression and function of transcription factors plays an important role in tumorigenesis (20,21).

c. Dietary fat and breast cancer risk: Overview. Epidemiological studies demonstrate a strong association between fat intake and breast cancer incidence with the highest breast cancer rates occurring in countries in which fat contributes 40% or more of total calories (22-25). Studies with a variety of rodent mammary tumor models, including transgenic mice, indicate that polyunsaturated fatty acids (PUFA), particularly linoleic acid, are potent tumor promoters (22-28). In addition to PUFA intake, excess caloric intake also acts as a mammary tumor promoter (22-27). Differences in key enzymes in energy metabolism between ad libitum and restricted fed animals indicates that dietary practices can induce biochemical and molecular alterations that may contribute to the mammary tumor promoting effects of diet (24,27). In contrast, results from some case-control and prospective cohort studies do not support a link between diet and breast cancer incidence (28-30). As a result, the "diet/breast cancer hypothesis" is highly controversial (17,27,28). This proposal will investigate a unique family of transcription factors (C/EBPs) as potential links between diet and mammary tumor promotion.

d. C/EBPs: rationale for investigation in diet/mammary tumorigenesis. C/EBPs have been implicated in the regulation of a variety of pathways that can determine cell fate: ie proliferation, differentiation or cell death (apoptosis) (1-10). The pleiotropic effects of C/EBP isoforms strongly support investigation of these unique transcription factors as links between diet and promotion of initiated mammary cells. C/EBP

isoforms are highly conserved DNA binding proteins that are expressed in a tissue-specific manner (1-10). C/EBP isoforms form homo- and heterodimers and exhibit significant amino acid homology with other "leucine zipper" DNA binding proteins such as c-fos, c-jun and c-myc (1). Aberrant expression of fos and myc genes can induce cell transformation (20,21).

1) C/EBP-alpha. C/EBP-alpha gene expression is confined to growth arrested tissue (5,7,8,10). C/EBP-alpha may mediate the acquisition of hormone responsiveness in terminally differentiated tissues such as adipose, liver and retina (5,7,8,31). Since the mammary gland is capable of repeated cycles of proliferation and differentiation throughout life we hypothesized and subsequently confirmed that C/EBP-alpha expression is low in mammary tissue (11). **The absence of C/EBP-alpha, or aberrant induction of C/EBP-beta and/or C/EBP-delta (see below) could provide a rationale for the unique susceptibility of mammary tissue to diet-induced tumor promotion.**

2) C/EBP-beta. C/EBP-beta binds to the same DNA sequences as C/EBP-alpha and is homologous with C/EBP-alpha in the leucine zipper region (3,4). Our preliminary data are the first to show high levels of C/EBP-beta mRNA levels in mammary-derived tissue (11). A rat homologue of C/EBP-beta is activated as an early component of the growth response of rat PC12 cells and is regulated by translocation from the cytoplasm to the nucleus (6). **Aberrant expression of C/EBP-beta, possibly diet-related, may alter C/EBP-beta cellular localization and influence growth control in initiated mammary cells.**

3) C/EBP-delta. C/EBP-delta binds to the same C/EBP consensus sequences as C/EBP-alpha and C/EBP-beta (3,4). We provide the first evidence that C/EBP-delta is a major C/EBP isoform expressed in mammary tumors (11). C/EBP-delta mRNA levels increase during the early stages of adipocyte differentiation *in vitro* and then decline during expression of the fully differentiated fat cell phenotype (3). This suggests that C/EBP-delta may regulate early response genes in differentiation (3).

e. MMTV/c-neu transgenic mice: rationale for transgenic models in breast cancer research (32,33).

Investigating the relationship between diet and mammary tumor promotion requires a model in which mutations are not induced by the initiating event. Genotoxic carcinogens induce numerous mutations in mitochondrial and nuclear DNA (34,35). Therefore, carcinogens, which have helped define the association between diet and mammary tumor promotion, are inappropriate for investigations of this association at the molecular level. MMTV/c-neu transgenics are a highly relevant model for human breast cancer because mammary tumors in MMTV/c-neu mice over express the c-neu protooncogene (12). Over expression of the c-neu protooncogene (HER2/NEU or ERBB-2 in humans) is a common abnormality in human breast cancer and is often associated with a poor prognosis (13,14). In addition, the majority of female MMTV/c-neu transgenics develop invasive mammary ductal adenocarcinomas, the most common form of human breast cancer (12-14). Finally, mammary tumors in MMTV/c-neu transgenic mice undergo a relatively high rate of metastasis, similar to human breast cancer (12-14).

f. Technical Objectives

Technical Objective #1: Investigate the influence of dietary fat (5% and 20% corn oil) fed ad libitum and at 20% caloric restriction (Tables 1 & 2) on C/EBP isoform **subcellular localization** in mammary tumors and nontransformed mammary tissue from MMTV/c-neu transgenic mice and age-matched mammary glands from nontransgenic controls (FVB/N).

Rationale: The transcriptional activity of C/EBP isoforms is controlled, at least in part, by cellular compartmentation (6). Diet may influence C/EBP function by affecting cellular compartmentation of C/EBP isoforms expressed in the mammary gland and this may alter growth-arrest or differentiation-inducing genetic programs.

Technical Objective #2: Investigate the influence of dietary fat (5% and 20% corn oil) fed ad libitum and at 20% caloric restriction on the **DNA binding activity** of mammary tumor nuclear extracts to a C/EBP consensus binding sequence.

Rationale: Changes in development and metabolic state influence C/EBP DNA binding activity (10). Diet may alter C/EBP function by altering C/EBP isoform binding to DNA consensus sequences.

6. BODY

a. General study design/Experimental methods. Female MMTV/c-neu transgenic mice and FVB/N nontransgenic controls (same strain as MMTV/c-neu mice) will be fed 4 experimental diets varying in fat content (5 vs 20% corn oil) and feeding regimen (ad libitum vs 20% caloric restriction) for 200 days.

b. Diet Rationale. The 5 and 20% corn oil diets fed ad libitum and at 20% caloric restriction have been extensively used in carcinogen initiated mammary tumor promotion studies (22-27). The 5% CO diet approximates a standard mouse diet, providing adequate EFAs and fat calories for mammary tumorigenesis (22-27). The 20% CO diet approximates fat consumption in Western countries (22-27).

Table 1.

Diet	f% corn oil	# mice/diet	Feeding
1	5	80*	ad libitum
2	20	80	ad libitum
3	5	80	80% of diet 1
4	20	80	80% of diet 2

* 40 MMTV/c-neu transgenic and 40 FVB/N nontransgenic controls/diet (Table 2) (80 total).

c. Mice . Female MMTV/c-neu transgenic mice (12) (Charles River, Wilmington, MA.) are randomly assigned to the 4 diets (160 total mice) for the 200 day study. Mice are killed 7 days after a mammary tumor detection (by palpation) and mammary tumors and noninvolved mammary glands harvested. The tumor incidence was expected to range from 10-60% (see below). Forty mice/treatment are required to detect a 30% difference in tumor incidence at alpha = 0.05 and beta = 0.10 (36). Forty weanling female nontransgenic control mice (FVB/N strain) were randomly assigned to the four diets (160 mice total).

d. Food intake and diets. All mice are individually housed. The food intake of the ad libitum fed group is determined by placing 10g of food pellets in each cage and weighing the remainder 24 hours later. The restricted fed mice are given 80% of the energy intake of the ad libitum fed groups. Diets (BioServ, Inc., Frenchtown, NJ) contain 5% and 20% corn oil (12.5% and 50% of calories from corn oil (CO) (60% linoleic acid). Diets are stored at 4 degrees C in the dark in covered containers. Energy restricted diets will be supplemented with vitamins and minerals to equalize the intake of these nutrients.

Table 2. Diet Composition

Ingredient	I	II	III	IV
Basal mix (g)	35	35	42	42
Glucose (g)	54	20	54	20
Corn oil (g)	5	20	5	20
approx. % cal from corn oil	13	50	13	50
approx. % cal from linoleic acid	8	30	8	30

Basal mix contains (g/35g basal): casein, 20.0; methionine, 0.3; AIN vitamin mix ((1.0); choline chloride, 0.2; AIN mineral mix, 4.0; and cellulose, 9.5. Grams of ingredients to equal 340 calories.

e. Mammary tumors (11). Mice will be sacrificed 7 days after tumor detection. Tumors will be dissected free of surrounding tissue, weighed, frozen in liquid nitrogen and stored at -80. A portion of selected tumors will be examined to verify mammary origin and tumor classification.

f. Mammary gland isolation (37,38). Mammary glands are isolated from the inner skin surface with the aid of a dissecting microscope. Isolated mammary tissue is dissociated by incubation in digestion buffer, loaded onto discontinuous Percoll gradients (20-80% in medium 199) and centrifuged at room temperature for 20 minutes at 2,220 rpm. The top layer (mammary fat cells) is discarded and the lower layers containing epithelial cells and fibroblasts are recovered for analysis.

g. Western blot (5,39). Mammary tumors and mammary epithelial cells will be isolated as described above, lysed in SDS-buffer and protein concentration determined. Samples are boiled and 50 micrograms of cellular protein is loaded onto a 10% SDS-polyacrylamide gel, electrophoresed and electroblotted to Hybond-ECL (Amersham, Arlington Heights, IL) overnight at 4 degrees. The filter is blocked overnight with buffer containing nonfat dry milk and probed with appropriate dilutions of rabbit antimouse C/EBP isoform antisera (purchased from Santa Cruz, Biotechnology, Inc. Santa Cruz, CA.). After washing, blots are developed with goat anti-rabbit IgG-horseradish peroxidase (Amersham, Arlington Heights, IL) . Western blots assess the steady state level of a specific protein.

h. Subcellular localization of C/EBP isoforms (6). Mammary tumors will be harvested, minced on ice and dissociated by collagenase treatment followed by gentle sieving through 50 and 100 mesh screens. The single cell suspension is pelleted, resuspended and dounced (20 strokes, loose pestle) in ice cold hypotonic buffer containing 10mM HEPES (pH=8), 5M KCL, 2mM MgCl2, 1mM DTT, 0.0005% leupeptin, 200 units Trasylol and 1mM PMSF. The nuclei are pelleted by centrifugation (2,000 x g, 5 minutes) and the presence of the relatively pure intact nuclei verified by microscopy. Nuclear preps are washed in hypotonic buffer and solubilized in SDS containing buffer. Nuclear and cytoplasmic C/EBP protein content is assessed by Western blot as described above.

i. Mobility Shift assay (6,17, 40, 41). (1) Nuclear extract preparation. Mammary tumors are homogenized in cold homogenization buffer (HB) containing 10mM HEPES, 25mM KCL, 0.15mM spermine, 0.5mM spermidine, 1mM EDTA, 2M sucrose, 10% glycerol, 1 mM DTT and 1mM PMSF. Homogenates are diluted and centrifuged 24,000 rpm for 30 minutes at -2 degrees C in an SW27 rotor. The nuclear pellet is repelleted, washed, and vortexed in a buffer containing 0.35M NaCl, 5mM Na-EDTA, 1mM DTT, 10mM HEPES plus proteinase inhibitors. The suspension is centrifuged at 10,000 x g for 15 minutes at 4 degrees C. The supernatant is frozen in 15% glycerol in liquid nitrogen.

(2) Band shift assay. A consensus C/EBP binding site (5'-TGC AGA TTG CGC AAT CTG-3') is end-labeling with 32P-gamma-ATP and T4 polynucleotide kinase. The end labelled C/EBP site (about 1 ng, 10,000 cpm) is mixed with the nuclear extract (10 micrograms of nuclear proteins) in 25 microliters final volume in solutions containing 25mM HEPES (ph=7.8) 50mM KCL, 0.1mM EDTA, 1mM DTT, 10% glycerol and 2 micrograms poly [d(I-C)]. After 30 minutes at room temperature loading buffer is added and the band-shift reaction is loaded onto a 4% low ionic strength acrylamide gel. After electrophoresis the gel is dried and exposed to x-ray film.

j. Results.

1) Final Body weights: Body weights of experimental mice fed the 4 diets for 200 days is presented in Figure 1. The results indicate that the dietary component of the study was effective. The body weights of mice fed ad libitum (Diets 1 & 2) were greater than mice fed the restricted caloric intakes (Diets 3 & 4). In addition, within the ad lib and restricted fed groups the high fat groups (Diets 2 & 4) were greater than the low fat groups (Diets 1 & 3). This difference was also statistically different. Although it was not expected that body weights would differ within caloric intake groups, this observation is consistent with reports suggesting that caloric efficiency may by higher in high fat feeding (23).

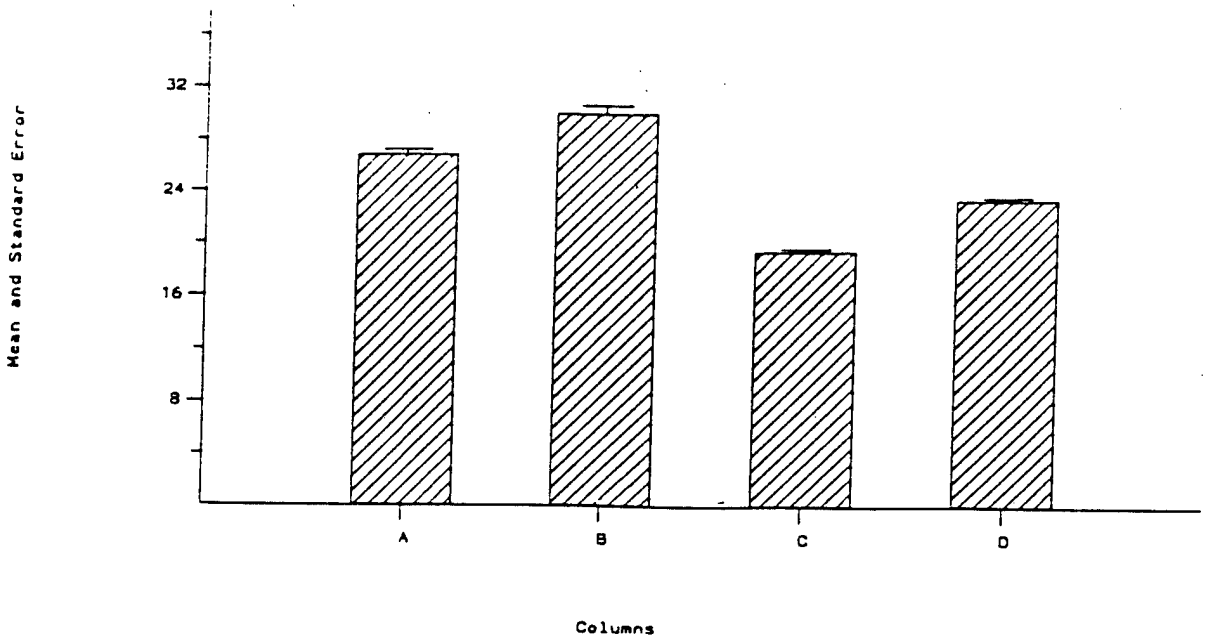

Figure 1. Final body weights of experimental mice fed 4 diets. Column A (diet 1, 5% corn oil, (CO) adlibitum); Column B (diet 2, 20% CO, ad libitum); Column C (diet 3, 5% CO, restricted fed); Column D (diet 4, 20% CO, restricted fed). Mean body weights: diet 1: 26.86 +/- 0.42; diet 2, 30.03 +/- 0.67; diet 3: 19.46 +/- 0.26; diet 4: 23.41 +/- 0.28 (mean +/- SEM)

Tukey-Kramer Multiple Comparisons Test
If the value of q is greater than 3.669 then the P value is less than 0.05.

Comparison	Mean Difference	q	P value
Diet 1 vs Diet 2	-3.160	7.006	*** P<0.001
Diet 1 vs Diet 3	7.409	15.907	*** P<0.001
Diet 1 vs Diet 4	3.459	7.393	*** P<0.001
Diet 2 vs Diet 3	10.569	23.030	*** P<0.001
Diet 2 vs Diet 4	6.619	14.356	*** P<0.001
Diet 3 vs Diet 4	-3.950	8.306	*** P<0.001

2) Mammary tumors: The influence of diet on mammary tumor incidence had not been extensively investigated using transgenic mice. This work is among a small number of studies that have used transgenic mice to investigate the link between diet and mammary tumorigenesis. Our laboratory was among the first to demonstrate that fat intake can influence mammary tumor incidence using MMTV/v-Ha ras transgenic mice (28). Since the cellular mechanisms underlying mammary tumor progression remain unknown, it is unclear if diet would alter mammary tumor incidence in other transgenic models. In this study we used MMTV/c-neu transgenic mice because these mice may represent a mammary tumor that is similar to human breast cancer.

The major difference between the MMTV/c-neu mouse used in these studies and the MMTV/v-Ha-ras mouse used in previous studies was the basal incidence of mammary tumors within the 200 day window of observation. The mammary tumor incidence in MMTV/v-Ha-ras mice ranged from 10-60%, which was what was also expected in the MMTV/c-neu mice. The observed incidence of mammary tumors in the

MMTV/c-neu mice ranged from 75-90%. At these high tumor levels we did not observe any differences across the dietary treatments.

Table 3

Diet	# mice with mammary tumors (40/group)
1	32
2	36
3	30
4	35

Although we did not observe diet-induced differences in mammary tumors this was not a technical objective of the study. Our goal was to investigate the relationship between diet and C/EBP isoforms expressed in mammary tumors.

3) Nuclear Localization of C/EBP isoforms: (Figures 2 A-H)

(a) C/EBP-beta: The C/EBP-beta gene is unique in that it is transcribed into a single mRNA that can be translated into multiple protein products with opposing biological activities (42,43). This is accomplished by a poorly understood "ribosome scanning" mechanism. The predominate C/EBP-beta "full-length" translation products are 35 and 32 kilodaltons (kd) in the mouse and 36 kd in human (42,43). The full-length C/EBP-beta translation products function as a transcription activators and are collectively called "LAP", for "liver-enriched activator protein. The third C/EBP-beta translation product is an in-frame, 20 kd protein which retains the DNA binding and bZIP regions but lacks the transactivation domain (42,43). This truncated C/EBP-beta translation product functions as a transcription inhibitor, hence the name, "LIP", for "liver-enriched inhibitory protein"). The C/EBP-beta multiple protein products result from "ribosome scanning", not proteolytic cleavage of a single full-length precursor (43). As a result of imperfect Kozak sequences in the first two AUGs, the ribosome may "scan" over these start sites and initiate translation at the downstream or third AUG, which is contained within a perfect Kozak initiator sequence (43). Initial reports suggested that this "ribosome scanning" mechanism was a random event, however, more recent data suggest a regulated process (43).

LAP & LIP bind to the same DNA consensus site, but LIP binds with higher affinity (42). Therefore, LIP may inhibit LAP transcriptional activation by occupying C/EBP binding sites on C/EBP-beta inducible promoters, or forming heterodimers with LAP that may function inefficiently as transcriptional activator complexes. LAP:LIP ratios of 20:1 or greater are common in many cell types, including mammary epithelial cells (44,45). This ratio is consistent with transcriptional activation by LAP. Lower LAP:LIP ratios (ie, 5:1 or less) are associated with LAP inhibition (44). LIP may also inhibit other bZIP protein in addition to LAP, but this has not been reported. The regulation of C/EBP-beta function in growth was initially described in PC12 cells, a cell line of neural origin (6). Initiation of the cell cycle in PC12 cells was associated with the rapid movement of C/EBP-beta (within 60 minutes) from the cytoplasm to the nucleus (6). This suggested that a major mechanism of regulation of C/EBP-beta, and possibly other C/EBPs, was through rapid changes in subcellular localization.

Because of the importance in the C/EBP-beta isoform expressed and the potential role of subcellular localization in the regulation of C/EBP-beta function, we carried out Western blot analyses of mouse mammary tumors (Figure 2A-C). Mammary tumors express both the LAP (32kd) and LIP (20kd) C/EBP-beta isoforms. The 35kd C/EBP-beta isoform also appears to be expressed in mammary tumors, however,

we cannot rule out the possibility that the slower mobility band is a phosphorylated form of LAP. Both isoforms are primarily localized to the nucleus in mouse mammary tumors (Fig. 2A-C). This is consistent with previous work in our lab in mouse mammary epithelial cell lines demonstrating that C/EBP-beta isoforms are primarily localized to the nucleus (46).

Figure 2A

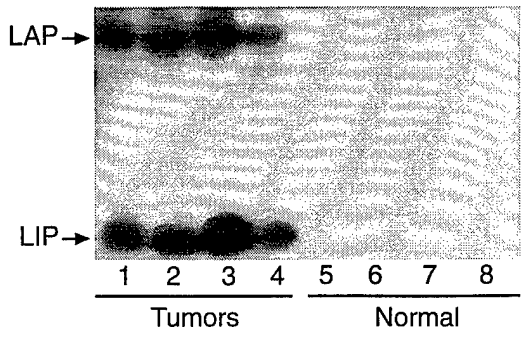

Figure 2B

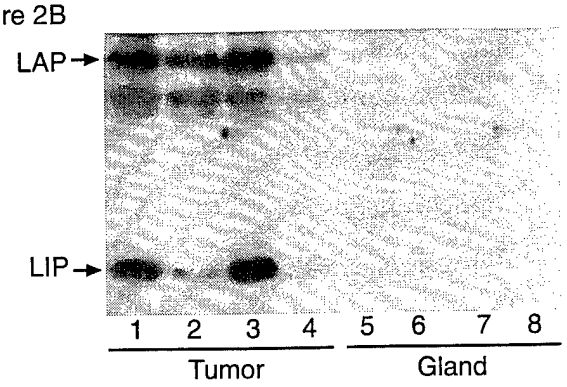

Figure 2C

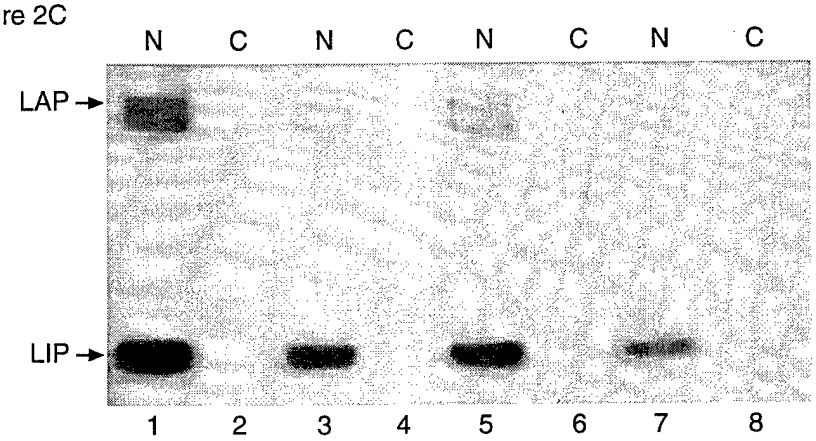

Figure 2. A-C. Mouse mammary tumors (MMTV/c-neu) express C/EBP-beta isoforms. Mammary tumors were harvested and lysates prepared from mice fed diet 1-4. Fig. 2A &B Diet/Lanes: diet 1/lanes 1,5; diet 2/ lanes 2,6; diet 3/lanes 3,7; diet 4/lanes 4,8. Fig. 2C: diet 1/lanes 1,2; diet 2/lanes 3,4; diet 3/lanes 5,6; diet 4/;lanes 7,8. The results from 12 different tumors (3/diet) are presented in Figure 2A-C. These results are representative of all tumors analyzed. Additional Western blot analyses of C/EBP-beta isoforms in mammary tumors and normal mammary gland are presented in Figure 3C-G. Western blot analysis was performed as described in materials and methods. N = nucleus, c = cytoplasm.

(b) C/EBP-delta: Our laboratory has reported a unique role for C/EBP-delta in mammary epithelial cell growth control (46,47). We found that C/EBP-delta is highly induced at the mRNA and protein level in quiescent (G0) mouse mammary epithelial cells in vitro (47). C/EBP-delta mRNA and protein levels decline rapidly after cell cycle induction. In addition, C/EBP-delta protein content is localized to the nucleus in cultured mammary epithelial cells (46). In vivo studies with lactating mouse mammary gland indicate that C/EBP-delta mRNA is rapidly and transiently induced during post weaning involution (48).

The data from mouse mammary tumors presented in Figure 2 D & E demonstrate that mouse mammary tumors from all diets produce significant quantities of C/EBP-delta. C/EBP-delta is

predominately localized to the nucleus in mammary tumors (Figure 2D & E). There were no apparent diet-induced differences in subcellular localization of C/EBP-delta. In contrast to mammary tumors, normal mammary gland may exhibit a more even distribution of C/EBP-delta protein between the nuclear and cytoplasmic compartments (Fig. 2D). Figure 2F compared nuclear isolates from mammary tumors (lanes 2-5) and normal mammary gland (6-9) from comparable diets. C/EBP-delta protein levels are detectable in all mammary tumors. In contrast C/EBP-delta is barely detectable in nuclear preps from normal mouse mammary glands. There was a tendency for the tumors from the restricted fed mice to have higher levels of C/EBP-delta protein. Although it is difficult to interpret this observation at this time it suggests that mammary tumors from restricted fed mice are expressing, C/EBP-delta, a gene that we have found to be highly expressed in growth arrested mammary epithelial cells (46). This suggests that mammary tumors from restricted fed mice may initiate, but not complete, a growth arrest response.

Figure 2D

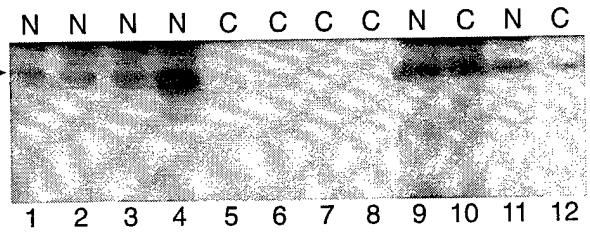

Figure 2E

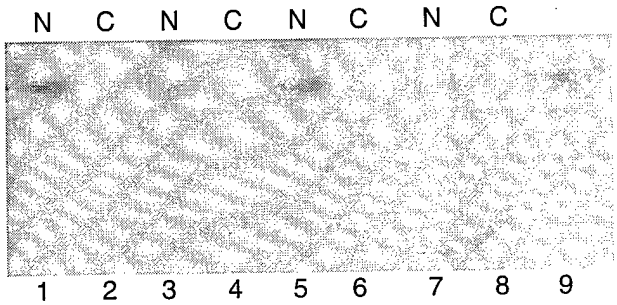

Figure 2F

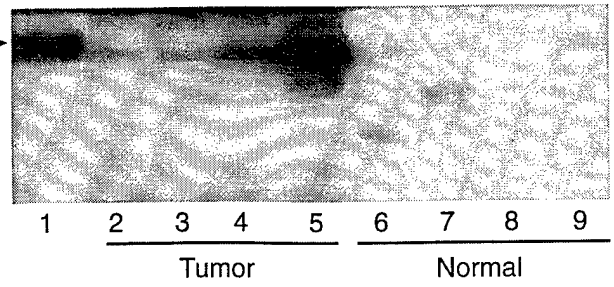

Figure 2 D-F. Mouse mammary tumors (MMTV/c-neu) and normal mouse mammary gland express C/EBP-delta. Mammary tumors and normal mammary glands were harvested, lysates prepared and Western blot analysis performed as described in Materials and Methods. The results from 12 tumors are presented in Figures 2D-F. These results are representative of all tumors analyzed. Fig. 2D Diets/Lanes: diet 1/ lanes 1,5; diet 2/lanes 2,6; diet 3/lanes 3,7; diet 4/lanes 4,8. Fig 2E Diets/lanes: diet 1/lanes 1,2; diet 2/lanes 3,4; diet 3/lanes 5,6, diet 4 /lanes 7,8. Fig. 2F diet/lanes: diet 2/lanes 2,6; diet 2/lane 3,7; diet 3/lanes 4,8; diet 4/lanes 5/9.
N = nucleus, c = cytoplasm. An in vitro expressed C/EBP-delta protein is used as a Western blot control in lane 9 (Fig. 2E) and lane 1 (Figure 2F).

(c) C/EBP-alpha: C/EBP-alpha has been implicated in the control of growth and differentiation in adipocytes and in liver (1-5). Specifically, C/EBP-alpha is elevated in quiescent adult liver and in growth arrested liver cells and adipocytes in culture (1-5). Over expression of C/EBP-alpha inhibits growth of liver

cells (5,6). In previous experiments were unable to detect C/EBP-alpha mRNA or protein in cultured mouse mammary epithelial cells (46,47). In mouse mammary gland, however, we detected low levels of C/EBP-alpha mRNA during early gestation and during the latter stages of involution (47). The significance of C/EBP-alpha in mammary gland biology is unknown. We were unable to detect C/EBP-alpha protein by western blot in mammary tumors or normal mouse mammary gland in the studies carried out in this project. To insure that our primary antibody was working we analyzed C/EBP-alpha protein in mouse liver, a tissue that is highly enriched in C/EBP-alpha. Like C/EBP-beta, C/EBP-alpha can be translated into multiple products, a 43kd full-length translation product and a 30kd truncated translation product. The full-length C/EBP-alpha contains the amino terminal transactivation domain, the truncated product lacks the transactivation domain. As a result, the truncated C/EBP-alpha translation product lacks the growth inhibition function that is retained in the full-length C/EBP-alpha translation product.

C/EBP-alpha was readily detectable in mouse liver. The unique observation that we made in this report, however, was reduced level of the full-length C/EBP-alpha translation product in the livers of ad libitum fed groups compared to the restricted fed groups (Figure 2G & H). This suggests that hepatic growth control by C/EBP-alpha may be compromised in ad libitum fed compared with restricted fed animals. As observed with C/EBP-beta and C/EBP-delta, C/EBP-alpha was localized to the nucleus.

Figure 2G

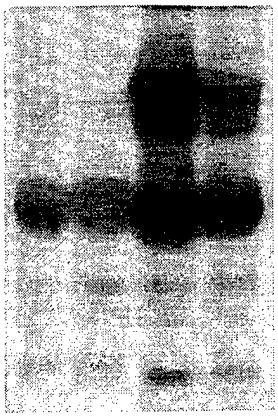

Figure 2H

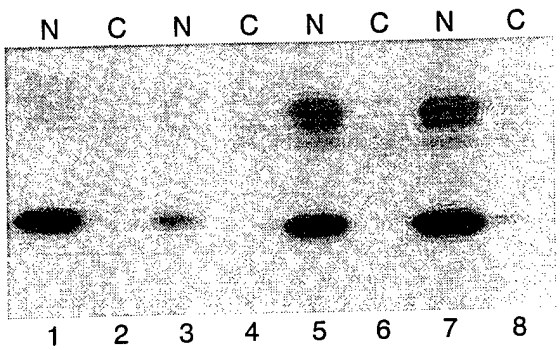

Figure 2 G & H. Mouse liver expresses C/EBP-alpha. Normal mouse livers were harvested, lysates prepared and Western blot analysis performed as described in Materials and Methods. The results from 8 livers are presented in Figure 2D & E. These results are representative of all livers analyzed. Diets/Lanes: diet 1/lane 1; diet 2/lane 2; diet 3/lane 3; diet 4/lane 4 (Fig.2G). Diets/Lanes: diet 1/lanes 1,2; diet 2/lanes 3,4; diet 3/lanes 5,6 diet 4/lanes 7,8 (Fig, 2H). N = nucleus, c = cytoplasm.

Protein loading was relatively even across all lanes as verified by Coomassie staining (data not shown). The amount of protein loaded in each lane for all Western blots was further verified by Western blot using antisera to alpha-actin (Figure 2I).

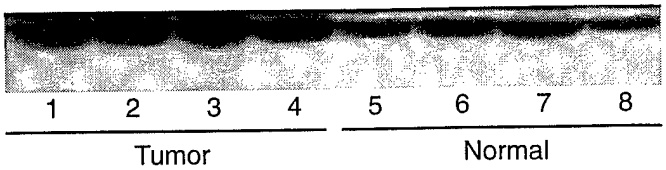

Figure 2 I. Mouse mammary tumors and normal mammary gland protein loading. Mouse mammary tumors and normal mouse mammary gland were harvested, nuclear lysates prepared and Western blot analysis performed (Materials and Methods). The results from 4 tumors and 4 normal gland isolates. These results are representative of all samples analyzed. Diets/Lanes: diet 1/1,5; diet 2/ 2,6; diet 3/3,7; diet 4/4,8.

4) C/EBP-beta: mammary tumor express high levels of the C/EBP-beta truncated translation product LIP (liver enriched inhibitory protein) (Figure 3A-F).

We initially observed that mammary tumors express both the full-length and truncated C/EBP-beta translation products at a ratio of 1:1 (Figure 2A-C). This was a novel finding that could be highly significant if confirmed in multiple tumors and in comparison with normal mammary tissue. The "normal" pattern of C/EBP-beta translation products in mouse mammary gland was analyzed over a lactation time course spanning from early gestation, through lactation and involution. Both C/EBP-beta and C/EBP-delta protein levels were assessed. The results suggested that normal mouse mammary gland expressed predominately the full-length LAP C/EBP-beta translation product in the virgin gland, and a ratio of 20:1 or greater of LAP:LIP throughout pregnancy, lactation and involution (Figure 3A). C/EBP-delta protein levels were relatively constant throughout the lactation time course in the mammary gland (Figure 3B).

We next carried out an extensive analysis of LAP:LIP ratios in mammary tumors and normal mammary gland tissue isolates from virgin female mice (Figure 3C-I). All mammary tumors analyzed, regardless of dietary treatment, express a LAP:LIP ration of 1:1 or less. This is true in 100% of tumors. In contrast, LAP and LIP were barely detectable in normal mammary gland isolates. Figure 3H demonstrates that relatively even amounts of cellular proteins were loaded onto gels as actin was detected at similar amounts in both tumors and normal mammary tissue isolates.

Figure 3

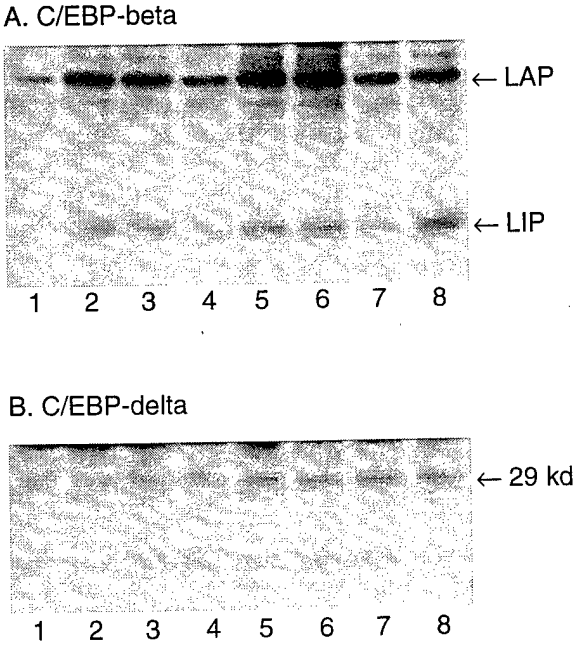

Figure 3 A & B. Normal mouse mammary gland expresses C/EBP-beta and C/EBP-delta during gestation, lactation and involution. Normal mouse mammary glands were harvested, nuclear lysates prepared and Western blot analysis performed (Materials and Methods). Lanes: 1: virgin gland, 2: gestation day 14, 3: gestation day 18, 4: postpartum day 1, 5: postpartum day 8, 6:postpartum day 14, 7: postpartum day 21, 8: postpartum day 23.

Figure 3C

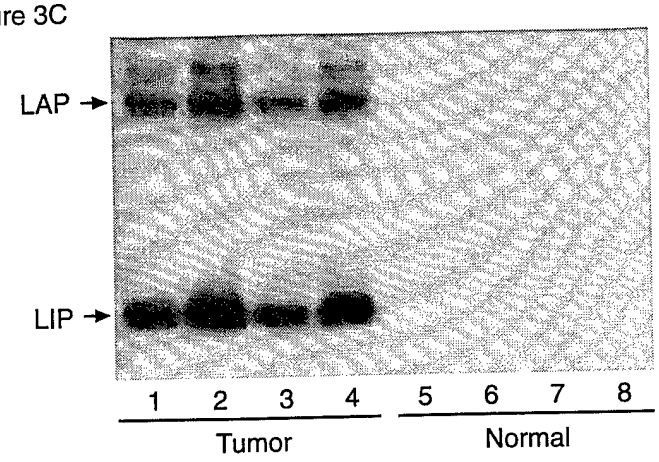

Figure 3F

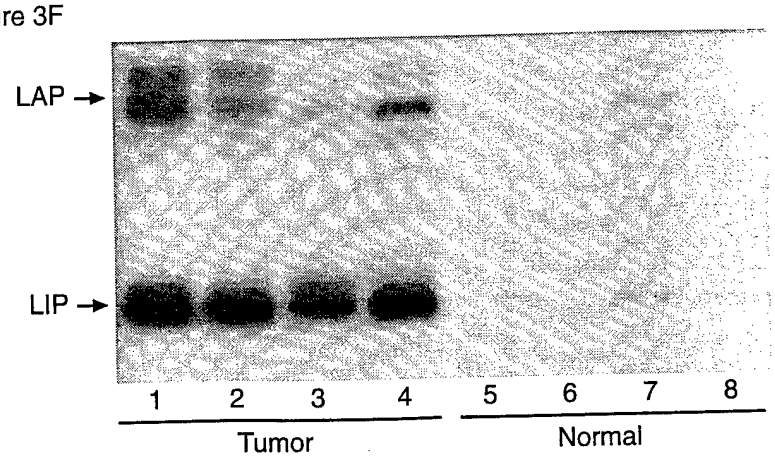

Figure 3D

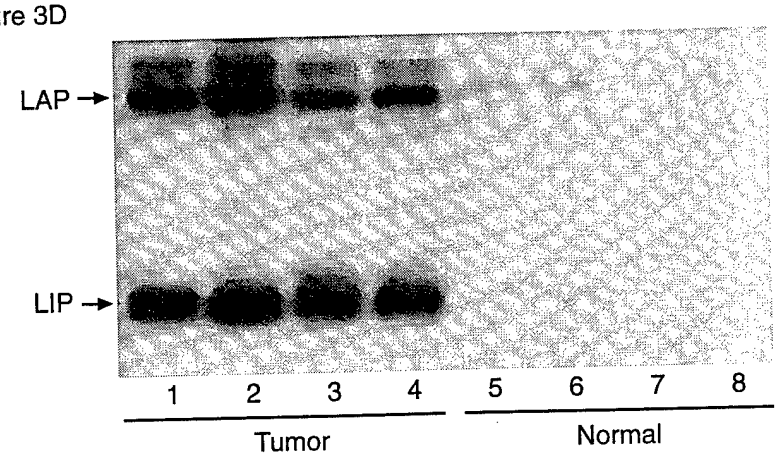

Figure 3G

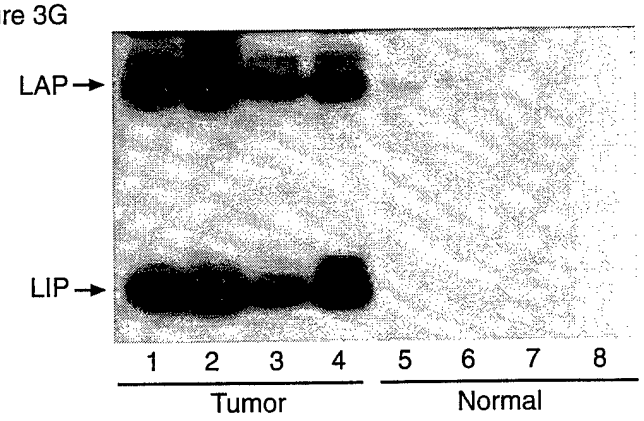

Figure 3E

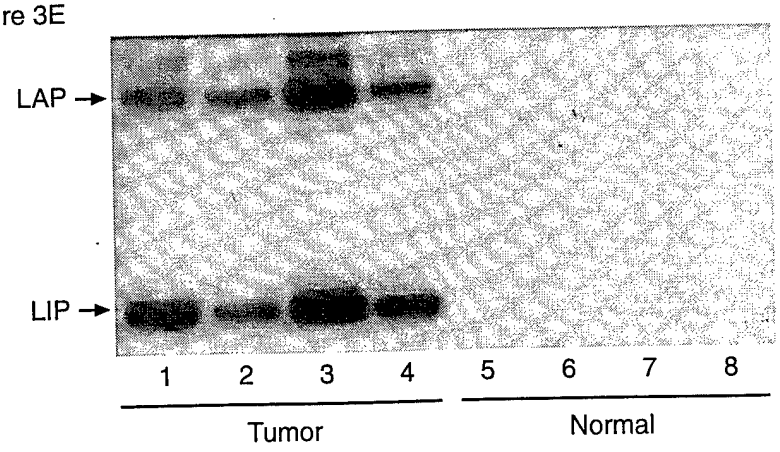

Figure 3H

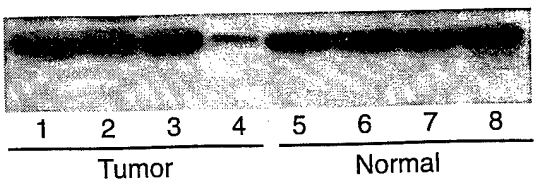

Figure 3. C-I. Mouse mammary tumors (MMTV/c-neu) and normal mammary gland express C/EBP-beta isoforms. Mammary tumors were harvested and nuclear lysates prepared and Western blots performed. Diet/Lanes: diet 1/lanes 1,5; diet 2/ lanes 2,6; diet 3/lanes 3,7; diet 4/lanes 4,8.The results are from 20 different tumors and 20 normal mice (5/diet).

5) Nuclear extracts: binding to C/EBP consensus site (Figure 4A-E).

Initial experiments were carried out to assess the capacity of nuclear extracts from mammary epithelial cell lines (COMMA D) to bind to a P32-radiolabelled C/EBP consensus site (Figure 4A). This binding can be dissociated by preincubation with C/EBP-delta antisera (Figure 4B), preimmmune sera does not interfere with C/EBP-consensus binding (Figure 4C). We next assessed binding to the C/EBP consensus site in nuclear extracts from mouse liver, mammary tumors and normal mammary gland (Figure 4D). Also included in this analysis was an in vitro transcribed and translated C/EBP-delta product to provide a control for binding of C/EBP-delta homodimers to the C/EBP consensus site. A nuclear lysate from 3T3 cells, a cell line that expresses moderate levels of C/EBP-delta (lane 2) was also added as a nuclear lysate control. Both liver and mammary tumors demonstrated significant binding to the C/EBP consensus site. No binding was detected in the normal mammary gland samples. Differences in binding between the liver and mammary tumor samples suggest that dimerization partners differ between liver and mammary tumors. In both tissue isolates there was some binding detected in the region that commigrated with the C/EBP-delta homodimers. However, binding that did not comigrate with the C/EBP-delta homodimers indicated the presence of other proteins bound to the C/EBP consensus binding site. In mammary gland, this additional binding was likely to involve C/EBP-beta (detected by western blot), CHOP10 and possibly fos, jun or even the CREB/ATF family.

Incubation of nuclear extracts with C/EBP-delta antisera interfered with the formation of the band shift complex in the tumor nuclear lysates, suggesting that C/EBP-delta may be a component of the dimerization complex that bound the C/EBP consensus site (Figure 4E, lanes 7-10). Interestingly the C/EBP-delta antisera appeared to supershift the C/EBP-delta homodimer formed in vitro transcription assay (Figure 4E lane 1 vs 6). The C/EBP-delta homodimer that is bound to the C/EBP consensus site is not affected by incubation with C/EBP-alpha antisera (Fig. 4E, lane 11), similarly, the shifted complex formed by mammary tumor nuclear extracts are also unaffected by incubation with the C/EBP-alpha antisera (Fig. 4E, 12-15). This is consistent with the results from the Western blots indicating that mammary tumors do not express C/EBP-alpha (data not shown).

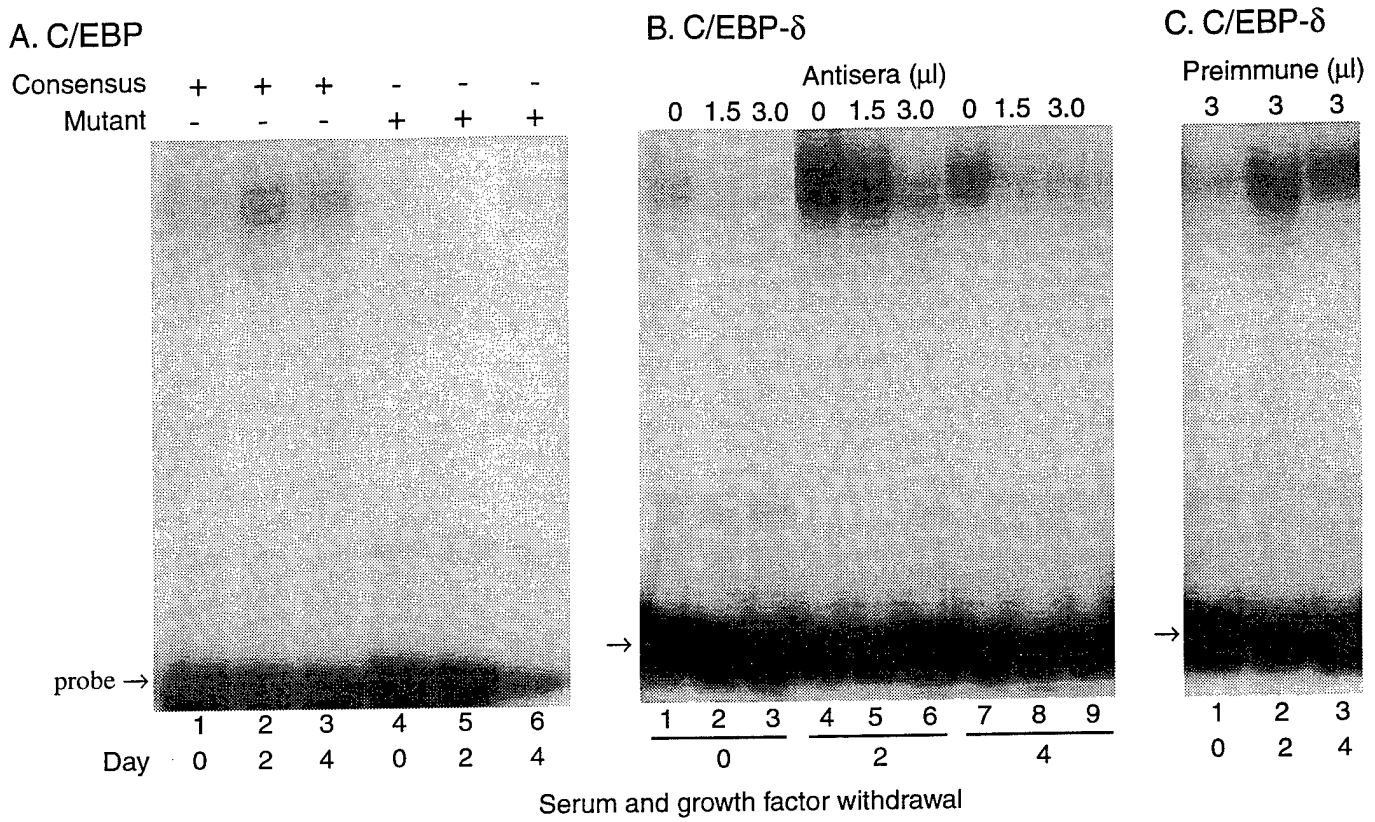

Fig 4. A-C. Binding to the C/EBP consensus sequence in nuclear lysates from cultured mouse mammary epithelial cells. Nuclear lysates were isolated from COMMA D mouse mammary epithelial cells under varying growth conditions and incubated with 32P-labelled C/EBP consensus binding site as described (Materials and Methods). Antisera used in antibody interference assays ("supershift assays") was purchased from Santa Cruz Biotechnology, Santa Cruz, CA).

Figure 4D

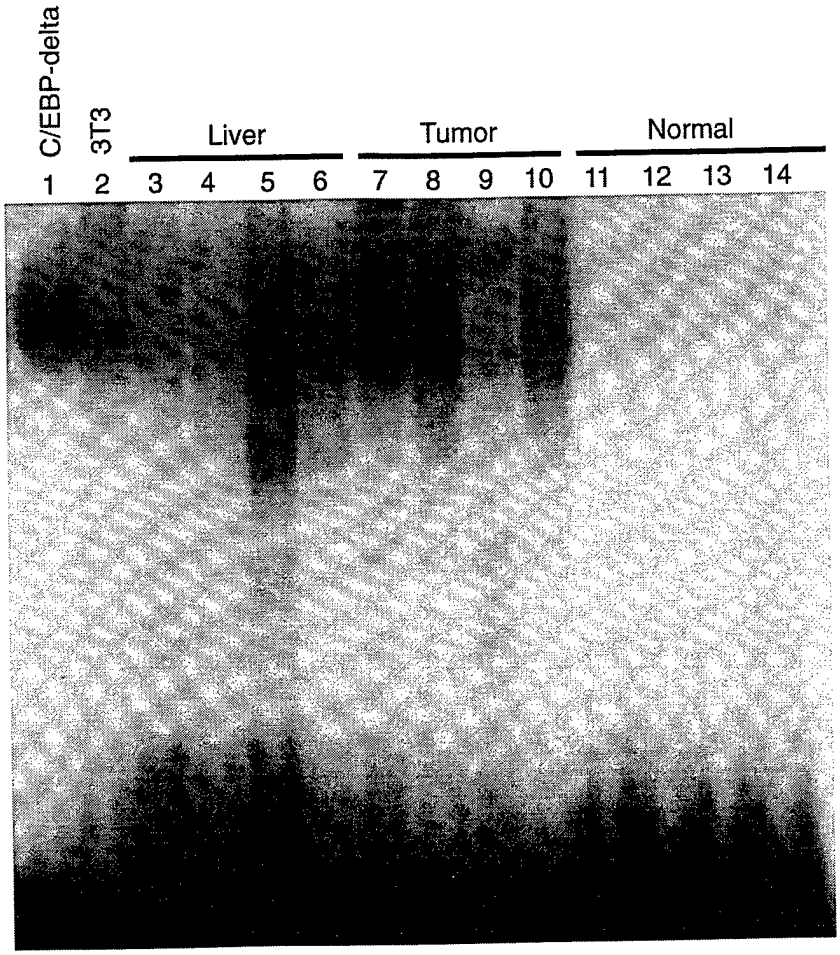

Figure 4D. Binding to the C/EBP consensus sequence in nuclear lysates from 3T3 cells, mouse liver, mammary tumors and normal mouse mammary gland. Nuclear lysates were isolated and incubated with 32P-labelled C/EBP consensus binding site as described (Materials and Methods). Diets/lanes: diet 1/lanes 3,7,11; diet 2/lanes 4,8,12; diet 3/lanes; 5,9,13; diet 4/lanes 6,10,14. Lane 1: In vitro expressed C/EBP-delta protein: C/EBP-delta homodimers; lane 2: 3T3 nuclear lysate.

Figure 4E

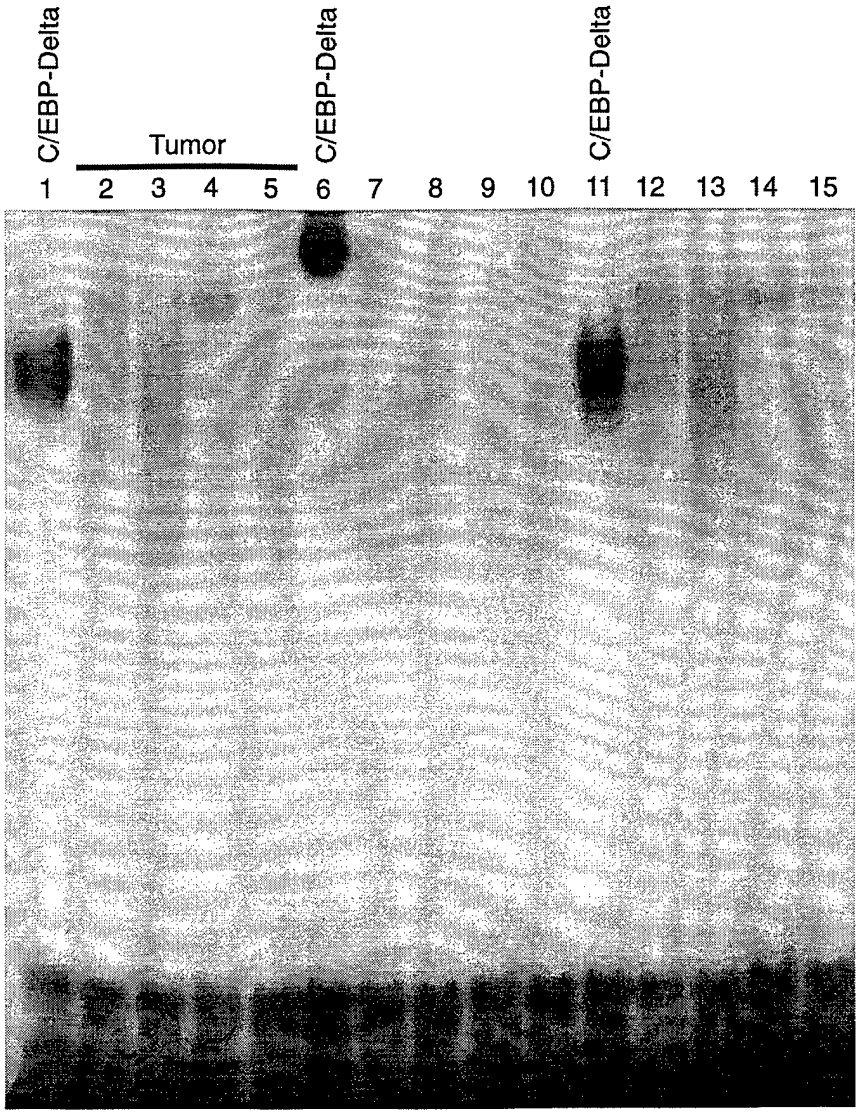

Figure 4E. Binding to the C/EBP consensus sequence in nuclear lysates from mouse mammary tumors is disrupted by incubation with C/EBP-delta antisera. Nuclear lysates were isolated from mammary tumors and incubated with 32P-labelled C/EBP consensus binding site as described (lanes 2-5), incubations were next carried out with C/EBP-delta antisera (lanes 7-10); incubations were carried out with C/EBP-alpha antisera (lanes 12-15)(Materials and Methods). Diets/lanes: diet 1/lanes 2,7,12; diet 2/lanes 3,8,13; diet 3/lanes; 4,9,14; diet 4/lanes 5,10,15. Lanes 1,6,11: In vitro expressed C/EBP-delta protein: C/EBP-delta homodimers.

6) Discussion

(a) Diet/Body weights and mammary tumor incidence:

Mouse body weights reflected the dietary treatments. The body weights of mice fed ad libitum were statistically greater than mice fed the restricted diets (Figure 1). The body weights of mice fed the high fat diet were statistically greater than mice fed equal caloric intakes in the form of a low fat diet. This is consistent with the hypothesis that fat, as a source of dietary energy, is utilized more efficiently than carbohydrate (23,24).

Despite differences in body weights we did not observe any differences among dietary treatments in mammary tumor incidence. This was somewhat unexpected, as previous experiments in our lab with another transgenic mouse model (MMTV/v-Ha-ras) resulted in diet-induced differences in mammary tumor incidence (11,28). The major difference between these two models is the tumor incidence in the MMTV/v-Ha-ras mice was about 10-60%, the incidence in the MMTV/c-neu mice in this experiment was 75-90%. This is somewhat misleading, however, as we have done other studies in which the mammary tumor incidence was less than 5% in MMTV/v-Ha-ras mice and the mamamry tumor incidence in MMTV/c-neu was about 50%. The bottom line is the tumor incidence appears to vary significantly in these models, at least at the present time. This may be due to breeding at commercial suppliers, where the genetic background may be changed to allow for coat color selection, increased litter size, disease resistance or other positive traits. It is also possible that there is drift in the expression of the transgenes over generations. I strongly believe that the transgenic model is the best way to go for these types of studies and the MMTV/c-neu is the most physiologically relevant model developed to date. It may take some time to sort out the variables in the models.

(b) Subcellular localization of C/EBP isoforms: Technical Objective #1.

This is the first examination of C/EBP isoform subcellular localization in an animal model. We found that C/EBP-beta translation products LAP and LIP were localized to the nucleus in mammary tumors. This also true for mammary epithelial cell lines COMMA D and HC11 (46). This is an interesting observation as previous reports have found that C/EBP-beta (NF-IL6) is regulated by changes in subcellular localization (6). This is not due to differences in techniques as we have observed the same results with different isolation techniques and in cultured mammary epithelial cells, normal mammary gland cells and mammary tumors. There were no differences across the dietary treatments in nuclear localization of C/EBP-beta.

C/EBP-delta was also predominately localized to the nucleus in mammary tumors, although about 20% was found in the cytoplasm. This is similar to results from experiments with cultured mouse mammary epithelial cells (46). In contrast, C/EBP-delta protein content was more evenly distributed between the nucleus and cytoplasm of normal mouse mammary gland and liver. The function of C/EBP-delta in growth regulation is unclear, but evidence from our lab suggests that C/EBP-delta plays a role in growth arrest (46). The relatively high levels of C/EBP-delta in mammary tumors suggests that the tumors may be engaging growth control mechanisms without effect, or, the growth control mechanisms are altered and have lost their efficacy.

C/EBP-alpha expression in mammary gland is controversial. C/EBP-alpha protein has been reported in lactating rat mammary gland (44). Although we have detected small amounts of C/EBP-alpha mRNA in mammary gland isolations (47), C/EBP-alpha protein has been difficult to detect in the mammary gland by Western blot. We also did not detect any C/EBP-alpha in mammary tumors, however, C/EBP-alpha was readily detectable by Western blot in mouse liver isolations. It is of interest that in mouse livers we were able to detect a diet-induced shift in C/EBP-alpha translation products. Livers from ad libitum fed mice

predominately expressed the truncated form of C/EBP-alpha. In contrast, livers from the restricted fed mice expressed both the full length and the truncated C/EBP-alpha translation products. Since the full length C/EBP-alpha translation product functions as a growth inhibitor and the truncated product does not, this observation may suggest an alteration in the capacity of ad libitum fed mice to regulate liver growth under some conditions. Under normal growth conditions, there is no difference in liver weight or appearance between ad libitum and restricted fed mice (data not shown). As observed with the other C/EBP isoforms, C/EBP-alpha was predominately nuclear in liver preparations.

(c) Binding to the C/EBP consensus site: (Technical Objective #2).

We determined that nuclear extracts derived from cultured mouse mammary epithelial cells bound to the C/EBP consensus site. These experiments were useful in developing the techniques of nuclear isolation, mobility shift assays and antibody interaction analysis (supershift assays). We were able to also demonstrate that nuclear extracts from mouse mammary tumors and livers bound to the C/EBP consensus site. These binding reactions resulted in different banding patterns in band shift analysis, indicating that the shifted complexes derived from the liver and mammary tumors were composed of different proteins. We next performed antibody interaction experiments to investigate the potential role of C/EBPs in these complexes. The results indicated that the shifted complexes contain C/EBP-delta and C/EBP-beta, but no C/EBP-alpha. The was essentially in agreement with the Western blot analysis. We did not observe any differences across dietary treatments.

These preliminary binding experiments demonstrate the ubiquitous binding of C/EBP isoforms from different tissue, both normal and transformed. Increasing evidence indicates that C/EBPs can bind to a wide variety of dimerization partners, including other C/EBPs, other leucine zipper proteins and even the retinoblastoma protein. Our initial experiments, based on information available in the early 1990,2 probably underestimated the complexity of binding reactions that occur. In addition, it is also becoming increasingly evident that C/EBPs can bind to a variety of DNA target sequences that can vary significantly from the originally described consensus sequences used in these experiments. Future experiments should screen potential C/EBP binding sites to determine if diet or tissue transformation influences C/EBP binding.

7. CONCLUSIONS

1. Body weights of mice fed calorically restricted diets were lower than ad libitum fed mice, reflecting the difference in caloric intake.

2. Body weights of mice fed high fat diets (20% corn oil) were greater than mice fed similar caloric intake of a low fat diet (5% corn oil). This is consistent with reports that suggest that fat may be a more efficient dietary caloric source than carbohydrate (23).

3. Diets did not alter mammary tumor incidence. This was due, at least in part, to the high percentage of MMTV/c-neu mice that developed mammary tumors (75% or greater). This suggests that diet may not play a large role in tumors that are initiated through alteration in the HER-2neu pathway.

4. C/EBPs (-beta and -delta) are predominately localized to the nucleus in mammary tumors. This appeared to be most significant for C/EBP-delta, where normal mammary gland exhibited a more even distribution between the cytoplasmic and nuclear compartments.

5. Mammary tumors express extremely high levels of a truncated C/EBP-beta translation product (LIP) compared to normal mammary gland. This novel observation suggests that mammary tumors may express a mutated C/EBP-beta mRNA, or, alterations in a signaling pathway may exist in mammary tumors that influence ribosome scanning mechanisms and result in the alteration in C/EBP-beta translation products.

6. Although diet did not affect C/EBP-beta translation products in mammary tumors, diet did affect C/EBP-alpha translation products in the liver. Restricted fed mice expressed both the full length (43kd) and the truncated (30kd) form of C/EBP-alpha. In contrast, ad libitum fed mice expressed high levels of the truncated C/EBP-alpha translation product. This finding may have important implications in the differing roles of C/EBP-alpha in growth control and differentiation in liver.

7. Nuclear extracts from mammary tumors bind to C/EBP consensus sites. The complex binding to the C/EBP consensus site appears to include C/EBP-beta and C/EBP-delta, but not C/EBP-alpha.

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